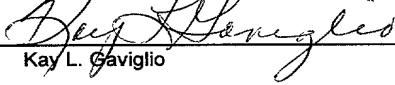


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Date: December 22, 1999

By: 
Kay L. Gaviglio

PATENT
Docket No. GC538-2

12/22/99
JC564 U.S. PTO

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09/470168
12/22/99

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

**UTILITY PATENT
APPLICATION TRANSMITTAL LETTER**

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, DC 20231

Dear Sir:

Enclosed for filing is the utility patent application of Matthew Grant Boston and Barbara A. Swanson for **METHOD FOR PRODUCING ASCORBIC ACID INTERMEDIATES.**

Also enclosed are:

- 9 Sheets of [] formal [X] informal Drawings, Figures 1-9
 Declaration and Power of Attorney--UNEXECUTED
 Assignment
 Sequence Listing
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 Statement of Sameness
 Information Disclosure Statement

The filing fee has been calculated as shown below:

	No. of Claims	Extra Claims	Rate	Fee
Basic Application Fee				\$760
Total Claims	62	MINUS 20 = 42	X \$ 18	756
Independent Claims	4	MINUS 3 = 1	X \$ 78	78
If Multiple Dependent Claims are presented, add \$270				270
Add Recording Fee of \$40.00 if Assignment document enclosed				-0-
Total Application Fee Due				\$1864

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Respectfully submitted,



Debra J. Glaister
Registration No. 33,888

Date: December 22, 1999

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Date: December 22, 1999

By:


Kay L. Gaviglio

ATTORNEY DOCKET NO. GC538-2

METHOD FOR PRODUCING ASCORBIC ACID INTERMEDIATES

RELATED APPLICATIONS

- This application is a continuation-in-part application of United States Serial
5 Number 09/218,700 filed December 22, 1998, which is hereby incorporated herein in
its entirety.

FIELD OF THE INVENTION

- The present invention relates to pathway engineering and in particular to
biocatalytic methods for the production of ascorbic acid intermediates. In particular,
10 the invention provides methods for the production of ascorbic acid intermediates in
non-fermentative systems.

BACKGROUND OF THE INVENTION

- L-Ascorbic acid (vitamin C, ASA) finds use in the pharmaceutical and food
15 industry as a vitamin and antioxidant. The synthesis of ASA has received
considerable attention over many years due to its relatively large market volume and
high value as a specialty chemical. The Reichstein-Grussner method, a chemical
route from glucose to ASA, was first disclosed in 1934 (Helv. Chim. Acta 17:311-
328). Lazarus et al. (1989, "Vitamin C: Bioconversion via a Recombinant DNA
20 Approach", Genetics and Molecular Biology of Industrial Microorganisms, American
Society for Microbiology, Washington D.C. Edited by C.L. Hershberger) disclosed a
bioconversion method for production of an intermediate of ASA, 2-keto-L-gulonic
acid (2-KLG, KLG) which can be chemically converted to ASA. This bioconversion
25 of carbon source to KLG involves a variety of intermediates, the enzymatic process
being associated with co-factor dependent reductase activity. Enzymatic co-factor
regeneration involves the use of enzymes to regenerate co-factors such as NAD+ to
NADH or NADP+ to NADPH at the expense of another substrate that is then
oxidized.

- There remains a need for economically feasible methods for the production of
30 ASA intermediates. In particular, when such methods involve the use of enzymatic

activities which require co-factor, it would be particularly desirable to have methods which provide for co-factor regeneration. The present invention addresses that need.

5 **SUMMARY OF THE INVENTION**

The present invention relates to the non-fermentative production of ASA intermediates, e.g., KDG, DKG and KLG, and ultimately their conversion to desired end products, e.g., erythorbate and ascorbic acid, from a carbon source in a biocatalytic environment, said environment referred to herein as a bioreactor. The
10 biocatalytic environment may comprise viable or non-viable host cells that contain at least one enzymatic activity capable of processing the carbon source to the desired intermediate. In one embodiment, the desired intermediate is purified from the bioreactor via electrodialysis prior to conversion to the desired end product. See Figure 2 for a schematic representation of the production of these intermediates and
15 products.

The present invention also relates to a non-fermentative process for the production of ASA intermediates wherein required co-factor is regenerated. The present invention is based, in part, upon the discovery that catalytic amounts of co-factor can be regenerated in a non-fermentative, or *in vitro*, method for the
20 production of KLG from a carbon source. In one embodiment of the present invention, the required co-factor is purified from the bioreactor via nanofiltration and reused.

When KDG is the desired ASA intermediate, the bioreactor is provided with a carbon source which is biocatalytically converted through at least one oxidative step
25 to KDG. In this embodiment, the host cell may comprise a mutation(s) in a gene encoding an oxidative enzymatic activity specific to oxidizing the KDG such that the KDG is not further converted to other intermediates or products.

When DKG is the desired ASA intermediate, the bioreactor is provided with a carbon source which is biocatalytically converted through at least one oxidative step
30 to DKG. Depending upon the host cell used, the host cell may comprise a mutation(s) in a gene encoding an oxidizing or reducing enzymatic activity such that DKG is not further converted to other intermediates.

When KLG is the desired ASA intermediate, the bioreactor is provided with a carbon source which is biocatalytically converted through at least one oxidative step and at least one reducing step to KLG. Depending upon the host cell used, the host cell may comprise a mutation(s) in a gene(s) encoding an oxidizing or reducing

5 enzymatic activity such that KLG is not further converted to other intermediates.

When the oxidative step and reducing step require co-factor, the method provides a means for co-factor regeneration.

In one embodiment, the host cells are recombinant and comprise at least one heterologous enzymatic activity. In one embodiment, the enzymatic activity is bound

10 to host cell membranes; in another embodiment, the enzymatic activity is in solution; in another embodiment, the enzymatic activity is soluble inside the cell; and in another embodiment, the enzymatic activity is immobilized. The process may be performed as a batch process or a continuous process. The host cells are preferably members of the family Enterobacteriacea and in one embodiment, the member is a

15 Pantoea species and in particular, Pantoea citrea. Pantoea citrea can be obtained from ATCC having ATCC accession number 39140, for example.

The host cells may be lyophilized, permeabilized, or otherwise treated to reduce viability or mutated to eliminate glucose utilization for cell growth or metabolism as long as the enzymatic activity is available to convert the carbon

20 source to the desired intermediate. The intermediates may be further processed to the end products of erythorbate or ASA.

Accordingly, in one aspect, the present invention provides a method for the production of DKG or KDG from a carbon source comprising enzymatically oxidizing the carbon source by at least one oxidative enzymatic activity to yield DKG or KDG.

25 In another embodiment, the process comprises oxidizing the carbon source by a first oxidative enzymatic activity to yield a first oxidative product and oxidizing said first oxidative product by a second oxidative enzymatic activity to yield KDG. In one embodiment, the first oxidative enzymatic activity is a GDH activity and the second oxidative enzymatic activity is a GADH activity. The host cell may further comprise a

30 mutation in the naturally occurring nucleic acid encoding a KDGDH activity, such that the KDG is not further oxidized. The KDG may be further converted to erythorbate.

Alternatively, the process may further comprise oxidizing KDG by a third oxidative enzymatic activity to yield DKG.

For production of KLG, if the carbon source is KDG or if a carbon source is converted to KDG, the method comprises the steps of enzymatically oxidizing the
5 KDG by at least one oxidative enzymatic activity to an oxidation product; and enzymatically reducing said oxidation product by at least one reducing enzymatic activity to 2-KLG. Alternatively, if DKG is the carbon source or if a carbon source is converted to DKG, DKG is converted to KLG by a reducing enzymatic activity.

The present invention provides a process for the non-fermentative production
10 of 2-KLG from a carbon source, wherein said process comprises the following steps in any order, enzymatically oxidizing the carbon source by at least one oxidative enzymatic activity to an oxidation product; and enzymatically reducing said oxidation product by at least one reducing enzymatic activity to 2-KLG. In one embodiment, said oxidative enzymatic activity requires an oxidized form of an enzymatic co-factor
15 and said reducing enzymatic activity requires a reduced form of said enzymatic co-factor and said oxidized form of said co-factor and said reduced form of said co-factor are recycled between at least one oxidizing step and at least one reducing step. In one embodiment, the oxidized form of the enzymatic cofactor is NADP+ and the reduced form of said enzymatic cofactor is NADPH. In another embodiment, the
20 oxidized form of said enzymatic cofactor is NAD+ and the reduced form is NADH. Other co-factors useful in the process of the present invention include ATP, ADP, FAD and FMN.

In one illustrative embodiment disclosed herein, the process comprises the following steps in any order and the steps may be occurring simultaneously and/or
25 continuously during the process: enzymatically oxidizing the carbon source by a first oxidative enzymatic activity to a first oxidation product; enzymatically oxidizing the first oxidation product by a second oxidative enzymatic activity to a second oxidation product; enzymatically oxidizing the second oxidation product by a third oxidative enzymatic activity to a third oxidation product; and enzymatically reducing the third
30 oxidation product by a reducing enzymatic activity to 2-KLG. In one embodiment, at least one of said first, second and third oxidative enzymatic activities requires an oxidized form of an enzymatic co-factor and said reducing enzymatic activity requires

a reduced form of said enzymatic co-factor and wherein said oxidized form of said co-factor and said reduced form of said co-factor are recycled between at least one oxidizing step and the reducing step.

In one embodiment, the process proceeds in an environment comprising
5 organic solvents and in another, the process proceeds in an environment comprising long polymers. In yet another embodiment, the process proceeds in an environment comprising a salt and within a range of salt concentrations.

The present invention also provides vectors and recombinant host cells comprising enzymatic activities which are used in the methods for producing the ASA
10 intermediates. In one embodiment, the host cell comprises heterologous nucleic acid encoding GDH obtainable from species including *T. acidophilum*, *Cryptococcus uniguttalatus* and *Bacillus* species and/or DKG reductase obtainable from *Corynebacterium* or *Erwinia*.

15 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a schematic representation of an in vitro process wherein NADP+ and NADPH are recycled between oxidation and reduction steps.

Figure 2 is a schematic representation of a pathway to ASA intermediates. Steps labeled A are enzymatic; steps labeled B are either enzymatic or chemical
20 conversions. In this representation, the enzyme that converts glucose (Glc) to GA is a GDH activity; the oxidative enzyme that converts GA to KDG is a GADH activity; the oxidative enzyme that converts KDG to DKG is a KDGDH activity and the reducing enzyme that converts DKG to KLG is DKGR activity.

Figure 3 illustrates the activity of reductase in the presence of 0-40% methanol at pH 7 and 30 °C.

Figure 4 illustrates Reductase activity in the presence of 0-50% ethanol at pH 7, 22 °C.

Figure 5 illustrates reductase activity at pH 7 in the presence of NaCl, KCl, CaCl₂, K₂SO₄, or potassium phosphate (KPi). Initial rates were measured over 1
30 min.

Figure 6 illustrates the activity of reductase remaining after incubation at pH 7 and 45 °C in the presence and absence of up to 500 mM 2-KLG.

Figure 7 shows the spectrophotometric measurement of NADPH in an in vitro cofactor recycling reaction. Absorbance was measured at 340nm. The initial absorbance reading was 0.7 before the enzymes were added. Additional aliquots of GDH were added at approximately 12 and 23 minutes.

5 Figure 8 shows the spectrophotometric measurement of NADPH in an in vitro cofactor recycling reaction. Absorbance was measured at 340mn. Enough NACL was added at approximately 5 minutes to bring the final concentration to 0.5M.

10 Figure 9 shows the reductase Km and relative Vmax for 2,5-DKG in the presence of increasing amounts of 2-KLG.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

The following abbreviations apply as used herein to glucose (G); D-gluconate (GA); 2-keto-D-gluconate (2KDG); 2,5-diketo-D-gluconate (2,5DKG or DKG), 2-keto-15 L-gulonic acid (2KLG, or KLG), L-idonic acid (IA), ascorbic acid (ASA), glucose dehydrogenase (GDH), gluconic acid dehydrogenase (GADH), 2,5-diketo-D-gluconate reductase (DKGR), and 2-keto-D-gluconate reductase (KDGDH).

As used herein, the term "non-fermentative" or "in vitro" refers to a biocatalytic process which exploits a cell's enzymatic activity. The cells may be non-viable or viable and not significantly growing. The cells may be genetically altered to eliminate their consumption of glucose and/or any intermediates produced. The in vitro process of the present invention encompasses the use of cell membranes which comprise enzymatic activity associated with the biocatalytic process, the use of permeabilized cells or lyophilized cells comprising the enzymatic activity associated with the biocatalytic process and the use of a host cell or host cell membranes or fragments in any form which provides the necessary enzymatic activity for the biocatalytic conversion of a carbon source to any of the ASA intermediates including but not limited to GA, KDG, DKG and KLG. The cell may be a recombinant cell which comprises heterologous nucleic acid encoding a desired enzymatic activity or a naturally occurring cell which comprises the desired enzymatic activity. The term "bioreactor" as used herein refers to the environment within which the non-fermentative or in-vitro process proceeds.

Many enzymes are only active in the presence of a co-factor, such as for example, NAD⁺ or NADP⁺. The term co-factor as used herein refers to a substrate secondary in nature to the enzymatic reaction, but vital to the enzymatic reaction.

As used herein, the term "co-factor" includes, but is not limited to NAD⁺/NADH;

5 NADP⁺/NADPH; ATP; ADP, FAD/FADH₂ and FMN/FMNH₂. The phrase "regeneration of co-factor" or "recycling of co-factor" within the in vitro system refers to the phenomenon of continual oxidation and reduction of the required co-factor through biocatalysis, such that the required co-factor is present in the appropriate form for enzyme catalysis to take place. In the present invention, regeneration of

10 co-factor provides an environment wherein a reduced form of a co-factor is available for a reducing enzyme and an oxidative form of the co-factor is available for an oxidizing enzyme. The present invention encompasses regeneration of co-factor between any enzymatic oxidation step and any enzymatic reducing step in the biocatalytic pathway from carbon source to the ASA intermediate, e.g. KLG. The

15 required co-factor may be present in catalytic amounts provided by the host cell environment or may be provided exogenously at the beginning of the bioreactor process in stoichiometric quantities in either an oxidized or reduced form.

As used herein, the term carbon source encompasses suitable carbon sources ordinarily used by *Enterobacteriaceae* strains, such as 6 carbon sugars,

20 including but not limited to glucose, gulose, sorbose, fructose, idose, galactose and mannose all in either D or L form, or a combination of 6 carbon sugars, such as sucrose, or 6 carbon sugar acids including but not limited to 2-keto-L-gulonic acid, idonic acid, gluconic acid, 6-phosphogluconate, 2-keto-D-gluconic acid, 5-keto-D-gluconic acid, 2-ketogluconatephosphate, 2, 5-diketo-L-gulonic acid, 2,3-L-

25 diketogulonic acid, dehydroascorbic acid, erythroascorbic acid and D-mannonic acid or the enzymatic derivatives of such as long as the carbon source is capable of being converted to an ASA intermediate such as, KDG, DKG and KLG, for example.

As used herein, the family "*Enterobacteriaceae*" refers to bacterial strains having the general characteristics of being gram negative and being facultatively anaerobic. Preferred *Enterobacteriaceae* strains are those that are able to produce 2,5-diketo-D-gluconic acid from D-glucose solutions. Included in the family of *Enterobacteriaceae* which are able to produce 2,5-diketo-D-gluconic acid from D-

glucose solutions are the genus *Erwinia*, *Enterobacter*, *Gluconobacter* and *Pantoea*, for example. Intermediates in the microbial carbohydrate pathway from a carbon source to ASA, include but are not limited to GA, 2KDG, 2,5DKG, 5DKG, 2KLG and IA. In the present invention, a preferred *Enterobacteriaceae* fermentation strain is a 5 *Pantoea* species and in particular, *Pantoea citrea*. Four stereoisomers of ascorbic acid are possible: L-ascorbic acid, D-araboascorbic acid (erythorbic acid), which shows vitamin C activity, L-araboascorbic acid, and D-xyloascorbic acid. As used herein, the term, ASA intermediate encompasses any product in the pathway to ASA including but not limited to KDG, DKG and KLG.

10 As used herein, the term "recombinant" refers to a host cell that contains nucleic acid not naturally occurring in the organism and/or to host cells having additional copies of endogenous nucleic acid recombinantly introduced. The term "heterologous" as used herein refers to nucleic acid or amino acid sequences not naturally occurring in the host cell. As used herein, the term "endogenous" refers to 15 a nucleic acid naturally occurring in the host.

As used herein, "nucleic acid" refers to a nucleotide or polynucleotide sequence, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be double-stranded or single-stranded, whether representing the sense or antisense strand. As used herein "amino acid" refers to 20 peptide or protein sequences or portions thereof.

As used herein the term "mutation" refers to any alteration in a nucleic acid such that the product of that nucleic acid is inactivated or eliminated. Examples of mutations include but are not limited to point mutations, frame shift mutations and deletions of part or all of a gene encoding an enzymatic activity, such as an oxidative 25 enzyme activity or a reducing activity. In one embodiment disclosed herein for producing KLG whereby co-factor is regenerated, nucleic acid encoding a membrane bound GDH activity is mutated thereby inactivating the endogenous GDH activity. In another embodiment, the 2-keto-D-gluconate dehydrogenase activity is inactivated thereby allowing for optimized production of the intermediate KDG.

30 The phrase "oxidative enzyme" as used herein refers to an enzyme or enzyme system which can catalyze conversion of a substrate of a given oxidation state to a product of a higher oxidation state than substrate. The phrase "reducing

enzyme" refers to an enzyme or enzyme system which can catalyze conversion of a substrate of a given oxidation state to a product of a lower oxidation state than substrate. Oxidative enzymes associated with the biocatalysis of D-glucose to KLG include among others D-glucose dehydrogenase, D-gluconate dehydrogenase and 5 2-keto-D-gluconate dehydrogenase. Reducing enzymes associated with the biocatalysis of pathway intermediates of ASA into KLG include among others 2,5-diketo-D-gluconate reductase (DKGR), 2-keto reductase (2-KR) and 5-keto reductase (5-KR). Such enzymes include those produced naturally by the host strain or those introduced via recombinant means. In one embodiment disclosed herein, 10 the process proceeds in a *Pantoea citrea* host cell having the naturally occurring membrane bound, non-NADP⁺ dependent GDH activity eliminated and a cytosolic NADP⁺ dependent GDH recombinantly introduced. In another embodiment, a heterologous nucleic acid encoding a reductase activity is introduced into the host cell. In a preferred embodiment, the reductase activity is obtainable from a 15 *Coryneform* species or an *Erwinia* species. As used herein, the term "pathway enzyme" refers to any enzyme involved in the biocatalytic conversion of a carbon source to an ASA intermediate, e.g., KDG, DKG and KLG.

The terms "isolated" or "purified" as used herein refer to a nucleic acid or protein or peptide or co-factor that is removed from at least one component with 20 which it is naturally associated. In the present invention, an isolated nucleic acid can include a vector comprising the nucleic acid.

It is well understood in the art that the acidic derivatives of saccharides, may exist in a variety of ionization states depending upon their surrounding media, if in solution, or out of solution from which they are prepared if in solid form. The use of a 25 term, such as, for example, idonic acid, to designate such molecules is intended to include all ionization states of the organic molecule referred to. Thus, for example, "idonic acid", its cyclized form "idonolactone", and "idonate" refer to the same organic moiety, and are not intended to specify particular ionization states or chemical forms.

30

DETAILED DESCRIPTION

The present invention relates to the biocatalytic production of ASA intermediates, e.g., KDG, DKG and KLG, from a carbon source in an in vitro or non-fermentative environment. Depending upon the intermediate being produced, the process may require the presence of enzymatic co-factor. In a preferred embodiment disclosed herein, the enzymatic co-factor is regenerated. Due to the cost of co-factor, it is highly advantageous to employ an in vitro process which allows for the regeneration of catalytic amounts of co-factor provided by the host cell environment or provided exogenously.

10 Non-Fermentative Production of ASA intermediates

The present invention provides a means for the production of ASA intermediates. Such intermediates can be further processed to ASA, ASA stereoisomers or other products such as erythorbate. In one preferred embodiment, KDG is the desired ASA intermediate produced, the bioreactor is provided with viable or non-viable *Pantoea citrea* host cells having a mutation in a gene encoding 2-keto-D-gluconate dehydrogenase activity as described herein in Example II. In this embodiment, the carbon source is biocatalytically converted through at two oxidative steps, see Figure 2, to KDG. In this embodiment, there is no need for co-factor regeneration.

20 When DKG is the desired ASA intermediate, the bioreactor is provided with viable or non-viable *Pantoea citrea* host cells and a carbon source which is biocatalytically converted through three oxidative steps, see Figure 2, to DKG. In this embodiment, there is no need for co-factor regeneration.

When KLG is the desired ASA intermediate, the bioreactor is provided with viable or non-viable *Pantoea citrea* host cells and a carbon source, such as glucose, which is biocatalytically converted through three oxidative steps, as shown in Figure 2 and one reducing step to KLG. In this embodiment, the reductase activity may be encoded by nucleic acid contained within the *Pantoea citrea* host cell or provided exogenously. In this embodiment, the first oxidative enzymatic activity requires an oxidized form of the co-factor and the reducing enzymatic activity requires a reduced form of co-factor. In a preferred embodiment disclosed herein, the *Pantoea citrea* cell is modified to eliminate the naturally occurring GDH activity and a

heterologous GDH obtainable from *T. acidophilum*, *Cryptococcus uniguttalatus* or *Bacillus* species and having a specificity for NADPH+ is introduced into the *Pantoea* cell in order to provide a co-factor recycling system which requires and regenerates one co-factor. This embodiment, provides a means for co-factor regeneration,
5 thereby eliminating the cost of continuously adding exogenous co-factor to the bioreactor for the production of KLG in *Pantoea* cells. In this embodiment, the host cell further comprises nucleic acid encoding a 2,5-DKG reductase activity or the 2,5-DKG reductase is added exogenously to the bioreactor.

In another embodiment for making KLG, the bioreactor is charged with
10 *Pantoea citrea* cells comprising nucleic acid encoding membrane-bound GDH, appropriate enzymes and cofactor, and gluconic acid is added which is converted to DKG. The reaction mixture is then made anaerobic and glucose is added. The GDH converts the glucose to GA, and the reductase converts DKG to KLG, while cofactor is recycled. When these reactions are completed, oxygen is added to
15 convert GA to DKG, and the cycles continue.

In vitro biocatalytic environment

A biocatalytic process of converting a carbon source to an ASA intermediate begins with a suitable carbon source used by *Enterobacteriaceae* strains, such as a
20 6 carbon sugar, including for example, glucose, or a 6 carbon sugar acid, such as for example, KDG. Other metabolite sources include, but are not limited to galactose, lactose, fructose, or the enzymatic derivatives of such. In addition to an appropriate carbon source, media must contain suitable minerals, salts, cofactors, buffers and other components known to those of skill in the art for sustaining cultures and
25 promoting the enzymatic pathway necessary for production of desired end-products. Preferred salts for the bioreactor are Na+, K+, NH4+, SO4- -, and acetate. The cells are first grown and for the non-fermentative process the carbon source utilized for growth is eliminated, the pH is maintained at between about pH 4 and about pH 9 and oxygen is present.

30 In the in vitro biocatalytic process, the carbon source and metabolites thereof proceed through enzymatic oxidation steps or enzymatic oxidation and enzymatic reducing steps which may take place outside of the host cell intracellular

environment and which exploit the enzymatic activity associated with the host cell and proceed through a pathway to produce the desired ASA intermediate. The enzymatic steps may proceed sequentially or simultaneously within the bioreactor and some have a co-factor requirement in order to produce the desired ASA

5 intermediate. The present invention encompasses an in vitro process wherein the host cells are treated with an organic substance, as described in Example V, such that the cells are non-viable, yet enzymes remain available for oxidation and reduction of the desired carbon source and/or metabolites thereof in the biocatalysis of carbon source to ASA intermediate. The present invention also encompasses an
10 in vitro process wherein the host cells are lyophilized, permeabilized by any means, spray-dried, fractured or otherwise treated such that the enzymes are available for the conversion of carbon source to ASA intermediate.

The oxidative or reducing enzymatic activities may be bound to a host cell membrane, immobilized, such as to a resin, for example AminoLink coupling gel
15 (from Pierce Chemical Co), to a polymer, or soluble in the bioreactor environment. In a preferred embodiment, at least one oxidative enzyme is membrane bound. The environment may proceed in an organic or aqueous system or a combination of both, and may proceed in one vessel or more. In one embodiment, the process proceeds in two vessels, one which utilizes oxygen and one which is anaerobic. For example,
20 the membrane bound enzymes that require oxygen (GDH, GADH, KDGDH) may be isolated from those enzymes that do not require oxygen (cofactor dependent GDH, cofactor dependent DKGR) allowing the use of a smaller volume containment vessel that requires oxygen, thereby reducing cost. The bioreactor may be performed in batch or in a continuous process. In a batch system, regardless of what is added, all
25 of the broth is harvested at the same time. In a continuous system, the broth is regularly removed for downstream processing while fresh substrate is added. The intermediates produced may be recovered from the fermentation broth by a variety of methods including ion exchange resins, absorption or ion retardation resins, activated carbon, concentration-crystallization, passage through a membrane, etc.

30 The bioreactor process may also involve more than one cell type, e.g., one cell may comprise the oxidative activities and a second cell may comprise the reducing activities. In another embodiment, the host cell is permeabilized or lyophilized (Izumi et al., *J. Ferment. Technol.* 61 (1983) 135-142) as long as the

necessary enzymatic activities remain available to convert the carbon source or derivatives thereof. The bioreactor may proceed with some enzymatic activities being provided exogenous and in an environment wherein solvents or long polymers are provided which stabilize or increase the enzymatic activities. In an embodiment disclosed herein, methanol or ethanol is used to increase reductase activity. In another embodiment, Gafquat is used to stabilise the reductase (see Gibson et al., US Patent 5,240,843).

In one illustrative bioreactor described herein, the host cell is a permeabilized *Pantoea citrea* cell provided D-glucose as a carbon source which undergoes a series of oxidative steps through enzymatic conversions. The oxidizing enzymes include GDH, GADH and DGDH and a reducing step which involves 2 DKGR (see United States Patent 3,790,444) to yield KLG. The KLG produced by a process of the present invention may be further converted to ascorbic acid and the KDG to erythorbate by means known to those of skill in the art, see for example, Reichstein and Grussner, *Helv. Chim. Acta.*, 17, 311-328 (1934).

Co-factor regeneration

One of the advantages of the process of the present invention lies in the regeneration of co-factor required by pathway enzymes. Examples of cofactor which can be used in the current process include but are not limited to NAD+/NADH; NADP+/NADPH; ATP; ADP, FAD/FADH² and FMN/FMNH².

In one embodiment of the invention, a carbon source is converted to KLG in a process which involves co-factor regeneration, as shown in Figure 1. In this enzymatic cofactor regeneration process, one equivalent of D-glucose is oxidized to one equivalent of D-gluconate, and one equivalent of NADP+ is reduced to one equivalent of NADPH by the catalytic action of GDH. The one equivalent D-gluconate produced by the GDH is then oxidized to one equivalent of 2-KDG, and then to one equivalent of 2,5-DKG by the action of membrane bound dehydrogenases GADH and KDGDH, respectively. The one equivalent 2,5-DKG produced is then reduced to one equivalent of 2-KLG, and the NADPH is oxidized back to one equivalent of NADP+ by the action of 2,5-DKG reductase, effectively recycling the equivalent cofactor to be available for a second equivalent of D-glucose oxidation. Other

methods of cofactor regeneration can include chemical, photochemical, and electrochemical means, where the equivalent oxidized NADP⁺ is directly reduced to one equivalent of NADPH by either chemical, photochemical, or electrochemical means. The amount of co-factor added exogenously to the bioreactor is between about 1µM to about 5mM and in a preferred embodiment, between about 5µM to about 1mM. As illustrated herein in the Examples, NaCl affects the Km for NADPH while KLG, a charged species, does not affect the Km. Therefore if NaCl is present in the bioreactor, more NADPH would be required to maintain an optimum rate. Furthermore, as disclosed in the Examples, most salts tested had an affect on thermal stability of the reductase. As will be appreciated by the skilled artisan, depending upon the conditions of the bioreactor, such as the temperature, the salt levels can be adjusted to provide a balance between thermal stability and acceptable rates. A co-factor added exogenously to an in vitro system may be added alone or in combination with other substances associated with biocatalytic conversion of a carbon source to an ASA intermediate. The present process encompasses the use of co-factor immobilized to a carrier, co-factor chemically altered, such as in attachment to a long polymer, and to the use of co-factor in an isolated or purified form.

The required co-factor may also be purified from the biocatalytic environment via nanofiltration and reused. Methods for using nanofiltration membranes for cofactor retention are described in, for example, Seelbach et al. (1997, Enzyme and Microbial Techhology, vol 20, pages 389-392).

Recombinant methods

Host cells

Any oxidative or reducing enzymes necessary for directing a host cell carbohydrate pathway into ASA intermediates, such as, for example, KDG, DKG or KLG, can be introduced via recombinant DNA techniques known to those of skill in the art if such enzymes are not naturally occurring in the host cell. Alternatively, enzymes that would hinder a desired pathway can be mutated by recombinant DNA methods. The present invention encompasses the recombinant introduction or mutation of any enzyme or intermediate necessary to achieve a desired pathway.

- In one embodiment of the present invention a carbon source, such as glucose, is converted to KLG through multiple oxidation steps and a reducing step. In this embodiment, the first oxidation step and the reducing step requires co-factor. The host cell is *Pantoea citrea*, the naturally occurring nucleic acid encoding glucose dehydrogenase (GDH) is mutated such that the dehydrogenase activity is eliminated and a heterologous GDH is introduced into the cell. The present invention encompasses a host cell having additional mutation of enzymes in the carbon flow pathway which affect production. For general techniques, see, for example, the techniques described in Maniatis et al., 1989, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, *Current Protocols in Molecular Biology*, Greene Publishing "Associates and Wiley Interscience, N.Y.

- In one embodiment of the present invention, nucleic acid encoding DKG reductase (DKGR) is recombinantly introduced into the *Pantoea* fermentation strain. Many species have been found to contain DKGR, particularly members of the *Coryneform* group, including the genera *Corynebacterium*, *Brevibacterium*, and *Arthrobacter*. In one embodiment of the present invention, 2,5-DKGR obtainable from *Corynebacterium* sp. strain SHS752001 (Grindley et al., 1988, Applied and Environmental Microbiology 54: 1770-1775) is recombinantly introduced into a *Pantoea citrea*. In another embodiment, 2,5 DKG reductase obtainable by *Erwinia herbicola* disclosed in United States Patent No. 5,008,193 to Anderson et al., is recombinantly introduced into *Pantoea citrea*.

Sources for nucleic acid encoding oxidative or reducing enzymes include the following:

ENZYME

glucose dehydrogenase

5

CITATION

Smith et al. 1989, Biochem. J. 261:973; Neijssel et al. 1989, Antonie Van Leauvenhoek 56(1):51-61

10

gluconic acid dehydrogenase

Matsushita et al. 1979, J.

15

2-keto-D-gluconic acid dehydrogenase

Stroshane 1977 Biotechnol. BioEng 19(4) 459

20

2-keto gluconate reductase

J. Gen. Microbiol. 1991, 137:1479

2,5-diketo-D-gluconic acid reductase

United States Patent Nos:
5,795,761; 5,376,544; 5,583,025;
4,757,012; 4,758,514; 5,008,193;
5,004,690; 5,032,514

Vector sequences

25 Expression vectors used in expressing the pathway enzymes, e.g., a dehydrogenase or reductase, of the present process in host microorganisms comprise at least one promoter associated with the enzyme, which promoter is functional in the host cell. In one embodiment of the present invention, the promoter is the wild-type promoter for the selected enzyme and in another embodiment of the 30 present invention, the promoter is heterologous to the enzyme, but still functional in the host cell. In one embodiment of the present invention, nucleic acid encoding the enzyme is stably integrated into the microorganism genome.

In a preferred embodiment, the expression vector contains a multiple cloning site cassette which preferably comprises at least one restriction endonuclease site 35 unique to the vector, to facilitate ease of nucleic acid manipulation. In a preferred embodiment, the vector also comprises one or more selectable markers. As used herein, the term selectable marker refers to a gene capable of expression in the host microorganism which allows for ease of selection of those hosts containing the vector. Examples of such selectable markers include but are not limited to 40 antibiotics, such as, erythromycin, actinomycin, chloramphenicol and tetracycline.

A preferred plasmid for the recombinant introduction of non-naturally occurring enzymes or intermediates into a strain of *Enterobacteriaceae* is RSF1010, a mobilizable, but not self transmissible plasmid which has the capability to replicate in a broad range of bacterial hosts, including Gram - and Gram+ bacteria. (Frey et 45 al., 1989, The Molecular biology of IncQ plasmids. In: Thomas (Ed.), Promiscuous Plasmids of Gram Negative Bacteria. Academic Press, London, pp. 79-94). Frey et

al. (1992, Gene 113:101-106) report on three regions found to affect the mobilization properties of RSF1010.

Transformation

5 General transformation procedures are taught in Current Protocols In Molecular Biology (vol. 1, edited by Ausubel et al., John Wiley & Sons, Inc. 1987, Chapter 9) and include calcium phosphate methods, transformation using DEAE-Dextran and electroporation. A variety of transformation procedures are known by those of skill in the art for introducing nucleic acid encoding a pathway enzyme in a
10 given host cell. The present process encompasses pathway enzymes produced by and purified from recombinant host cells and added exogenously into the in vitro environment as well processes wherein the pathway enzyme, either heterologous or endogenous to the host cell, is expressed by an actively growing host cell or present in the membrane of a non-viable host cell. A variety of host cells can be used for
15 recombinantly producing the pathway enzymes to be added exogenously, including bacterial, fungal, mammalian, insect and plant cells. Plant transformation methods are taught in Rodriguez (WO 95/14099, published 26 May 1995).

In a preferred embodiment of the process, the host cell is an *Enterobacteriaceae*. Included in the group of *Enterobacteriaceae* are *Erwinia*,
20 *Enterobacter*, *Gluconobacter* and *Pantoea* species. In the present invention, a preferred *Enterobacteriaceae* fermentation strain is a *Pantoea* species and in particular, *Pantoea citrea*. In another preferred embodiment, the host cell is *Pantoea citrea* comprising pathway enzymes capable of converting a carbon source to KLG. The present invention encompasses pathways from carbon source to KLG through
25 any intermediate in the microbial carbohydrate pathway capable of using a carbon source to produce KLG, going through intermediates including but not limited to GA, 2KDG, 2,5DKG, 5DKG, and IA. In one embodiment, nucleic acid encoding the pathway enzyme is introduced via a plasmid vector and in another embodiment, nucleic acid encoding a pathway enzyme is stably integrated into the host cell genome.
30

Identification of Transformants

Whether a host cell has been transformed can be detected by the presence/absence of marker gene expression which can suggest whether the
35 nucleic acid of interest is present. However, its expression should be confirmed. For

example, if the nucleic acid encoding a pathway enzyme is inserted within a marker gene sequence, recombinant cells containing the insert can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with nucleic acid encoding the pathway enzyme under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the enzyme as well.

Alternatively, host cells which contain the coding sequence for a pathway enzyme and express the enzyme may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

Additionally, the presence of the enzyme polynucleotide sequence in a host microorganism can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes, portions or fragments of the enzyme polynucleotide sequences.

Assay conditions

Methods for detection of ASA intermediates, ASA and ASA stereoisomers include the use of redox-titration with 2,6 dichloroindophenol (Burton et al. 1979, J. Assoc. Pub. Analysts 17:105) or other suitable reagents; high-performance liquid chromatography (HPLC) using anion exchange (J. Chrom. 1980, 196:163); and electro-redox procedures (Pachia, 1976, Anal. Chem. 48:364). The skilled artisan will be well aware of controls to be applied in utilizing these detection methods.

Recovery of Intermediates

Once produced, the ASA intermediates can be recovered and/or purified by any means known to those of skill in the art, including, lyophilization, crystallization, spray-drying, and electrodialysis, etc. Electrodialysis methods for purifying ASA and
5 ASA intermediates such as KLG are described in for example, United States patent number 5747306 issued May 5, 1998 and United States patent number 4767870, issued August 30, 1998. Alternatively, the intermediates can also be formulated directly from the bioreactor and granulated or put in a liquid formulation.

The manner and method of carrying out the present invention may be more
10 fully understood by those of skill in the art by reference to the following examples, which examples are not intended in any manner to limit the scope of the present invention or of the claims directed thereto. All references and patent publications referred to herein are hereby incorporated by reference.

15

Examples

Example I

This example describes the method for producing a *Pantoea citrea* host cell having a mutation in the naturally occurring GDH.

20 Cloning of glucose dehydrogenase gene (GDH) from *Pantoea citrea*: The glucose dehydrogenase gene was cloned by polymerase chain reaction (PCR). Two primers were used in the PCR: 5'AGGGAGTGCTTACTACCTTATCTGCGGTATA3' and 5'CGCTAGCTGTGCAATCCATTGATTTGCACA3'. After the PCR, a DNA product of about 2 kb was cloned in the vector, pGEM-T (Promega), and the recombinant E.
25 *coli* with the correct DNA insert was identified and the clone was designated as pRL. The DNA insert was analyzed by DNA sequencing and its sequence was found to be 60-70% identical to the published DNA sequences of a GDH of a strain of *Pantoea citrea*.

30 Generation of a deleted GDH gene by the insertion of chloramphenicol resistance gene:

To generate the deletion mutant of the GDH gene in *Pantoea citrea*, a recombinant copy of the gene to be deleted was first generated by the introduction of a selectable marker, chloramphenicol resistance gene (CAT). The in vitro generated copy was introduced into the *Pantoea citrea* and allowed to recombine with the wild-type copy through homologous recombination. The pRL DNA was then analyzed by digestion with various restriction enzymes. Two SmaI cleavage sites located about 700bp apart within the GDH encoding DNA were found. The pRL was digested with

Smal to remove the 700 bp fragment which was then replaced with a Smal digested 1.05 kb DNA containing the chloramphenicol resistance gene to generate the recombinant plasmid, designated as pRLcm4. The method used to generate pRLcm4 were standard techniques used by those of skill in the art. The GDH-CAT encoding sequence from pRLcm4 was further transferred to a plasmid, pGP704. The DNA encoding the GDH-CAT cassette was removed from pRLcm4 by the combined digestion of restriction enzymes AatII and Spel. The cohesive ends of the digested DNA were removed by the treatment of T4 DNA polymerase in the presence of deoxynucleotide triphosphate mixtures. The GDH-CAT cassette was 5 then ligated with the EcoRV digested pGP704. Recombinant plasmid of pGP704 containing the GDH-CAT cassette was identified and designated as p704RLcm.

10

Introduction of the deleted GDH gene into the chromosome of Pantoea citrea:
Plasmid p704RLcm was introduced into wild-type *Pantoea citrea* by electroporation. The transformed cell was first plated in agar plates containing 12.5 ug/ml of 15 chloramphenicol and resistant colonies were observed. To differentiate the true deletion mutant (which should display chloramphenicol resistant phenotype) from cells which simply harbors the plasmid p704RLcm, the chloramphenicol resistant colonies were screened against ampicillin, another antibiotic resistance marker carrier by p704RLcm. Ampicillin sensitive clones were identified. Several clones 20 which had the right phenotype (chloramphenicol resistant and ampicillin sensitive) were characterized by biochemical assays and all exhibited GDH negative phenotype. DNA blot analysis also confirmed that the wild-type GDH gene was replaced with the deleted copy.

25 **Example II**

Example II describes the method for producing a host cell having a mutation in the naturally occurring 2-Keto-D-gluconate dehydrogenase (E3).

2-Keto-D-gluconate dehydrogenase (EC1.1.99.4) from *Gluconobacter melanogenus* is purified according to the procedure of McIntire et al., (McIntire, W., 30 Singer, T.P., Ameyama, M., Adachi, O., Matsushita, K., and Shinagawa, E. *Biochem. J.* (1985) 231, 651-654) and references therein. The purified protein is digested with trypsin and chymotrypsin or other proteases to produce peptide

fragments which are separated by HPLC or other techniques. Individual peptides are collected and sequenced. From the sequence, DNA probes are synthesized which will anneal to the corresponding sequence in the host organism or a related organism's genome. Using standard PCR techniques, larger fragments of the
5 desired gene are amplified, purified and sequenced. These fragments are used to hybridize to the gene and allow for cloning and sequencing of the entire gene. Once the sequence is known, the gene is deleted as described for D-gluconate dehydrogenase (GDH) in Example 1

Other methods to reduce or eliminate 2-keto-D-gluconate dehydrogenase
10 include inhibitors (organic acids such as citrate and succinate are reported to inhibit 2-keto-D-gluconate dehydrogenase; Shinagawa, E. and Ameyama, M. Methods in Enzymology (1982) 89, 194-198), and changes in pH or temperature.

The enzyme can be assayed for activity or loss of activity using the assays described in Shinagawa and Ameyama.
15

Example III

Example III illustrates a method for producing KLG in Bioreactor where co-factor is regenerated.

20 Materials and Methods

Cell permeabilization

400 ml of *P. citrea* cells having a mutation in the naturally occurring membrane bound GDH was grown to 80 OD (600nm) in 10 g/L gluconate, and mixed with 16 ml of a mixture of 10% toluene and 90% acetone for 3 minutes at 22
25 C. The permeabilized cells were then centrifuged for 10 minutes at 9000 rpm, and the resulting cell pellet was washed with 400 ml of 50 mM tris, pH 7. The washings were repeated twice more to ensure removal of residual organic solvent.

Charging of the reactor

30 The 400 ml of permeabilized cells in 50 mM tris, pH 7 from above were placed into a one liter glass vessel equipped with a stirrer, temperature control, oxygen delivery tube, base delivery tube, a sample port, and oxygen and pH probes.

200 ul of MAZU antifoam (BASF) was added to the solution to control excess foaming, pressurized air was fed to the vessel, the temperature was brought to 28 C, and the stirrer was turned on to rotate at 1200 rpm until the oxygen probe read over 60% saturation. 16 grams of crystalline glucose and 4 grams of crystalline Na

5 gluconate were then added to a final concentration of 10 g/L gluconate and 40 g/L glucose. The mixture was allowed to react until all the gluconate had been converted to DKG. The glucose level was maintained above 20 g/L. Due to the cell permeabilization, minimal amounts of glucose entered into non-productive cellular metabolism. pH was maintained at 7 by the controlled addition of 50% NaOH

10 throughout.

Addition of the soluble enzymes and cofactor

Once the gluconate was converted to DKG, 2000 units each of cofactor dependent GDH and DKG reductase (for DKGR, one unit is equal to one OD absorbance change per minute when measured at 340nm) were added, along with 400 uM NADP+. The reactor was stirred, fed air, and maintained at 28 C as above. Periodic additions of glucose were made throughout the run to ensure constant substrate supply for both of the cofactor dependent enzymes.

20 Results

A bioreactor experiment was performed with non-purified reductase A:F22Y/A272G (US Patent No. 5,795,761), in the form of a crude extract from E. coli. T. acidophilum GDH and NADP+ were purchase in purified form from Sigma. GA to DKG rates were greater than 10 g/L/hr. Initial 2KLG formation rates were greater than 10 g/L/hr. Integrated rate over the first six hours was over 5 g/L/hr. Cofactor appeared to be stable over the first 6 hours, and predominantly in the reduced form. The total turnover number was 537 (215 mM 2KLG / 0.4 mM NADP+). During the first six hours, the intermediates GA and DKG never went above 4 g/L. The run was stopped 6.5 hours after the initial cell charge, and a wind down phase of low agitation at 22 C was run overnight. The final titer of KLG was about 42 g/L.

Aliquots were removed during the course of the bioreactor incubation. These aliquots were first spun in a microfuge to pellet the cells. To assay for remaining reductase activity, 25 microliters of sample supernatant were added to a solution composed of 910 ul buffer (50 mM bis-tris, pH 7), 20 ul DKG (70 mg/ml) and 250 uM NADPH. Reductase activity was measured by monitoring the loss of absorbance at 340 nm for 1 min. GDH activity was measured by adding 25 ul of sample to a solution containing 520 ul buffer, 150 ul NaCl (1 M), 200 ul urea (8 M), 50 ul glc (1 M) and 60 ul NADP+ (5 mM), and monitoring increase in absorbance at 340 nm for 1 min. Both the reductase and the GDH showed full activity throughout the course of the bioreactor experiment.

Example IV

This Example illustrates the production of KDG in an in-vitro bioreactor.

Cells containing membrane-bound D-glucose dehydrogenase and D-gluconic acid dehydrogenase activities but not 2-keto-D-gluconate dehydrogenase activity are grown and harvested. One example of such a cell is *Pantoea citrea* which has a mutation in the 2-keto-D-gluconate dehydrogenase enzyme, and is grown and treated as in Example III. The cells are permeabilized as described in Example III. Glucose (crystalline or in solution) is added in aliquots or continuously. The pH is maintained by controlled addition of a concentrated NaOH solution. The glucose is converted to D-gluconic acid and then KDG. Product formation is monitored by analyzing aliquots on a suitable HPLC system. Product is recovered by removing the cells by centrifugation and concentrating or removing the remaining liquid.

Example V

This example illustrates that the addition of organic solvents increases reductase activity 1-2 mg of DKG, 250 uM NADPH, F22Y/A272G reductase A and enough 50 mM bis-tris buffer, pH 7, to bring the final volume to 1 ml is added to a cuvette. Reductase activity is measured by monitoring the decrease in absorbance at 340 nm. The amount of reductase added typically produces a change in absorbance of 0.1-0.2 OD/min at room temperature or 30 °C. Under the same conditions, aliquots of methanol or ethanol were added to the solution and reductase activity measured.

Reductase activity in the presence of various amounts of methanol at 30 °C is shown in Figure 3, and activity in the presence of ethanol at 22 °C is shown in Figure 4.

As shown in the Figures, reductase activity is increased in the presence of certain amounts of methanol or ethanol. Optimal concentrations range between 10
5 and 25% of the organic solvent.

GDH from *T. acidophilum* has a small decrease in activity when it is incubated with 10% methanol (assay conditions are 50 mM Tris, pH 7, 12.5 mM D-glucose, 250 uM NADP+, in 1 ml. Activity is monitored by the increase in absorbance at 340 nm). Permeabilized cells were incubated with 15% MeOH and
10 gluconic acid. The activities of D-gluconic acid dehydrogenase and 2-keto-D-gluconic acid dehydrogenase were not significantly affected by the addition of methanol as monitored by product formation (HPLC analysis).

The addition of methanol or ethanol to a complete bioreactor reaction would increase reductase activity. Losses in the GDH activity or other components could
15 be overcome by adding more GDH or cells.

Example VI

Example VI illustrates the reductase activity in the presence of Gafquat and
20 PEG8000.

Reductase was incubated with 250 uM NADPH, 1-2 mg/ml DKG, and 0, 0.7% and 2.8% Gafquat (ISP Technologies, Inc.) or 0.5% PEG8000 in 1 ml (50 mM bis-tris buffer, pH 7) at 30 °C. Reductase activity was measured as in Example VI. As shown in Table 1, the addition of Gafquat increases reductase activity by 80%
25 compared to activity without Gafquat. PEG8000 increases reductase activity approximately 15%.

Polymer	% Added to Final Solution	% Activity with No Additive
Gafquat	0.7-2.8	180
PEG8000	0.5	115

Table 1. Increase of reductase activity in the presence of Gafquat or PEG8000.

30

Example VII

Example VII illustrates the reductase activity in the presence of salt.

Reductase A F22Y/A272G activity was measured in the presence of varying amounts of different salts. The assay consisted of adding reductase to a solution (1 ml final volume) containing 250 uM NADPH, DKG (1-1.5 mg/ml), 50 mM bis-tris buffer, pH 7.0, and varying amounts of potassium phosphate, NaCl, KCl, K₂SO₄ or CaCl₂. All reactions were done at 30 °C. The results are shown in Figure 5.

As shown in Figure 5, reductase activity stays the same or slightly increases when incubated with up to 100 mM NaCl or KCl. Activity then drops as salt concentrations are increased to 250 mM. Reductase activity drops in concentrations of CaCl₂ or potassium phosphate of 20 mM or more.

The reductase binding constant (K_m) for NADPH in the presence of 200 mM NaCl was determined using standard biochemical techniques (Fersht, A. "Enzyme Structure and Mechanism" (1977) W. H. Freeman and Company). The reactions were done in pH 7 bis-tris buffer containing approximately 1.5 mg/ml DKG at 30 °C and varying amounts of NADPH. The K_m for NADPH in the presence of 200 mM NaCl was found to increase 10-40 fold over the K_m determined without NaCl. The maximal rate (V_{max}) in salt was similar or slightly increased over the no-salt V_{max}. One way to reduce the effect of salt on reductase activity is to increase the concentration of NADPH until it is at or above the K_m under those conditions.

Alternatively, charged species including KLG could be removed.

Example VIII

Example VIII illustrates the stability of reductase A F22Y/A272G in presence of salts/product.

The reductase thermal stability was greatly increased in the presence of salts. Reductase was tested in one of the following ways. In the first case, reductase was added to buffer (50 mM bis-tris, pH 7) in the presence and absence of varying amounts of 2-KLG (0-500 mM). These solutions were then aliquoted (40 ul) into 1.5 ml eppendorf tubes. The tubes were then placed in a 45 °C water bath and removed at set intervals. The reductase was then assayed for remaining activity using the standard reductase activity assay. The results are shown in Figure 6.

As shown in Figure 6, reductase does not significantly lose any activity under these conditions in the presence of 500 mM 2-KLG. However, reductase incubated with

only buffer loses approximately half of its activity in 10 min. Intermediate 2-KLG concentrations give partial stabilization.

Reductase was incubated for 10 min in the presence of buffer (50 mM bis-tris, pH 7, or 25 mM MOPS, pH 7), 0.5M NaCl, 0.5M KCl, 0.5M NH₄Cl, 0.5M K₂SO₄ and 0.1M NaCl at pH 7 and 45 °C. As shown in Table 2, below, little activity is lost in the presence of these compounds, while reductase with buffer alone lost almost half of its activity. These compounds clearly stabilize the reductase. Lower and higher levels of these compounds should also stabilize the reductase.

10

Reductase Sample	% Remaining activity after 12 min incubation	% Remaining activity after 10min incubation
Buffer		25-40
0.5 M NaCl		100
0.5 M KCl		100
0.5 M NH ₄ Cl		100
0.5 M K ₂ SO ₄		100
0.1 M NaI	80-85	
100mM NADPH		80-90
200mM K ₂ PO ₄		65-75
100mM K ₂ SO ₄		90-100

Table 2. Reductase activity after incubation for 10 min at room temperature or 45 °C. Activity was measured using the standard assay.

15

A comparison between stabilization of 2-KLG and NaCl was done. The temperature incubation was done at 45.4 °C in 25 mM MOPS, pH 7. A concentration of 20 mM 2-KLG or 20 mM NaCl was used. Time points were assayed at 0, 5 and 10 min. The results are listed below in Table 3. As shown, the same amount of NaCl stabilizes the reductase more so than 2-KLG.

20

Conditions	% Activity, 0 min	% Activity, 5 min	% Activity, 10 min
20 mM NaCl	100	72	59
20 mM 2-KLG	100	51	29

In Table 3 all % are +/- 10%.

At 46.6-46.9 °C, the half-lives for reductase in the presence of 0-400 mM NaKLG have been determined. This temperature was selected in order to determine all of the half-lives at the same temperature. The buffer is 25 mM MOPS, pH 7.0. Aliquots are removed and assayed for remaining activity. The thermostability half-life measurements were performed as follows: a 450 μ l sample containing buffer, reductase and 2KLG (where used) was placed in an eppendorf tube and heated in a water bath. Eight or nine aliquots were removed over the time course, which varied from 10 to 30 minutes. Each aliquot was put on ice and assayed in duplicate at the end of the experiment. The activities were plotted time vs. remaining activity. The K_f 5 was determined b fitting the line using a computer graphing program for solving an exponential decay. This value is then used to calculate the half-life (Fersht et al., 10 1977, Enzyme Structure and Mechanism, W.H. Freeman and Co.). The results are shown in Table 4 below.

NaKLG conc.	0 mM	100 mM	200 mM	300 mM	400 mM
Half-life (min)	3.5 +/- 0.5	5.5 +/- 1	7.5+/- 1.5	10 +/- 3	18.5+/- 3

15 As the results in Table 4 show, NaKLG clearly stabilizes the reductase, and this stabilization is concentration dependent.

This data show that increasing amounts of salt can stabilize reductase. Suitable salts which may be used in the bioreactor include ammonium sulfate, 20 sodium acetate, ammonium acetate, ammonium chloride, sodium sulfate, potassium phosphate, sodium phosphate, sodium chloride, , KCl, NH₄Cl, K₂SO₄ and NaI. One of skill in the art would recognize that the optimum range of salt would be temperature dependent. Therefore, in the bioreactor, either the temperature or salt concentration 25 or both could be modified to achieve the desired stability of the reductase. At lower temperatures under which a typical bioreactor would be run, less salt would have to be used to provide the same amount of stabilization of the reductase as shown in Table 4.

Example IX

30 Example IX illustrates a method for measuring NADPH/NADP⁺ ratio and reaction equilibrium.

Reduced cofactor (NADPH) has a strong absorbance at 340 nm, while oxidized cofactor (NADP⁺) does not absorb at that wavelength. Therefore, if the two cofactors are mixed together, the amount of NADPH present can be determined by the absorbance at 340 nm. If the amount of NADP⁺ originally added is also known,
5 the ratio of the two cofactors can then be easily determined. This method can be used to measure how the addition of various components to a reaction, such as a cofactor recycling reaction, affects the reaction equilibrium.

A 1 ml reaction was set up in a cuvette at room temperature. The reaction consisted of buffer (50 mM bis-tris, pH 7), 5 mg glucose, 5 mg 2,5-DKG, 100 uM
10 NADPH, 100 uM NADP⁺, reductase and glucose dehydrogenase (GDH). The enzymes were added last to initiate the reaction, and the cofactor levels were monitored at 340 nm. After equilibrium was reached (Figure 7), an additional aliquot of GDH was added. Very quickly the equilibrium shifted to favor more NADPH being present. Addition of more GDH gave the same response.

15 Another 1 ml incubation was set up as above. After it reached equilibrium, 29 mg of NaCl was added to it to give a final concentration of 0.5 M NaCl. As shown in Figure 8, this dramatically shifted the equilibrium to favor the presence of NADPH.

Example X

20 Example X illustrates cofactor recycle reactions

Cofactor recycling reactions were performed by adding reductase, GDH, glucose, 2,5-DKG and NADP⁺ into a reaction vessel. Additionally, purified 2-KLG was added to some reactions to assess the reaction in the presence of product.
25 These reactions were sustained to produce gluconic acid and 2-KLG. Cofactor was recycled between NADP⁺ and NADPH by the action of the two enzymes. Aliquots were periodically removed and analyzed by HPLC for the presence of substrates and products. The reaction was sustained for at least 20 hours at room temperature.

Reactions as small as 3 ml were done. In a reaction, reductase, GDH, 10 mg/ml glucose and 10 mg/ml lyophilized 2,5-DKG were added to 50 mM bis-tris
30 buffer. 2-KLG was added to some incubations at a concentration of 75 mg/ml. The reaction was initiated at room temperature by the addition of NADP⁺ (400 uM). The solution pH was maintained between pH 6-7.5 by the addition of small amount of NaOH. Aliquots of glucose and 2,5-DKG were added periodically. At the end of a

day, the reaction was placed at 4 °C overnight. The following morning it was warmed to room temperature, the pH adjusted, and the reaction continued. Small aliquots were removed and injected on HPLC. By comparison to a standard, the amounts of gluconate and 2-KLG made could be calculated. In a typical reaction, at 5 least 60% of the glucose was converted to gluconate, and at least 60% of the 2,5-DKG converted to 2-KLG.

Example XI

Example XI illustrates NaCl kinetics.

10 When NaCl (100 mM or greater) was added to a standard reductase assay containing 250 uM NADPH and 10-20 mM DKG, the reductase rate decreased. By doing kinetic analysis, it was found that sodium chloride increases the Km of the reductase for the cofactor NADPH. As more NaCl is added, the Km increases. If a subsaturating amount of NADPH is used, the reductase appears to be inhibited by 15 NaCl. To counteract this effect, more NADPH was added to the reaction, such that it is several-fold above the Km. The mode of inhibition appears to be competitive.

The Km for DKG in the presence of NaCl was determined using standard techniques. The NADPH concentration used for each measurement was adjusted to at least 3 times above its Km at each NaCl concentration.

20

[NaCl], mM	Km for NADPH (mM)	Km for DKG (mM)
0	4-9	6-14
100	30-100	6-14
200	130-230	6-14
400	260-360	6-14

Example XII

Example XII shows 2-KLG kinetics.

25 The Km for DKG in the presence of 2-KLG was determined using standard biochemical techniques. The amount of 2-KLG was varied from 0 to 150 mM in pH 7 buffer. The Km for DKG under pH 7 conditions was 10-12 mM. As the concentration of 2-KLG increases, the Km for 2,5-DKG decreases. For example, the Km for DKG at 150 mM 2-KLG is 2-4 mM. The reaction rate also decreases, and experiences a 2-4-fold decrease when KLG concentration increased from 0 to 150 mM. This behavior is consistent with uncompetitive inhibition.

The Km for NADPH in the presence of 100 mM 2-KLG was determined to be 4-9 mM. This was done using standard biochemical techniques at pH 7, with a concentration of 2,5-DKG greater than 14 mM, and NADPH concentrations bracketing the Km value.

5 Therefore, the presence of increasing amounts of KLG in a bioreactor environment would be expected to reduce reductase activity and slow down the overall reaction rate. Two ways to overcome this phenomenon would be the KLG recovery, e.g. by electrodialysis, or alternatively by adding more reductase to the bioreactor.

10

Example XIII

Example XIII illustrates the synthesis of 2,5-DKG.

15 *P. citrea* cells are incubated with 150 mM sodium gluconate in an appropriate buffer: 25 mM bis-tris or 25 mM MOPS is used at pH 6. 25 mls of the cells, buffer and substrate are added to a 125 ml Erlenmeyer flask with baffles and incubated at 28 °C with approximately 250 rpm shaking. After 16-24 hours, the flask is monitored for formation of 2,5-DKG by HPLC analysis and activity assay. The cells are spun down and the supernatant removed. The material is sterile filtered, and can be stored at 4 °C or frozen. Alternatively, the material can be lyophilized to a solid.

20

WE CLAIM:

1. A process for the non-fermentative production of KDG or DKG from a carbon source comprising, enzymatically oxidizing the carbon source by at least one oxidative enzymatic activity to yield KDG or DKG .
5
2. The process of Claim 1 wherein said KDG is further converted to erythorbate.
3. The process of Claim 1 comprising oxidizing the carbon source by a first oxidative
10 enzymatic activity to yield a first oxidative product and oxidizing said first oxidative product by a second oxidative enzymatic activity to yield KDG.
4. The process of Claim 3 wherein said first oxidative enzymatic activity is a GDH activity and said second oxidative enzymatic activity is an GADH activity.
15
5. The process of Claim 1 that proceeds in an environment comprising host cells.
6. The process of Claim 5 wherein said host cell is non-viable.
20
7. The process of Claim 5 wherein said host cell is viable.
8. The process of Claim 5 wherein at least one oxidative enzyme is bound to said host cell membranes.
25
9. The process of Claim 1 wherein at least one oxidative enzymatic activity is in solution.
10. The process of Claim 8 wherein said host cell comprises a mutation in the naturally occurring nucleic acid encoding a KDGDH activity.
30
11. The process of Claim 5 wherein said host cell is an member of the family Enterobacteriaceae.

12. The process of Claim 11 wherein said member is a *Pantoea* species.
13. The process of Claim 1 wherein at least one oxidative enzymatic activity
5 immobilized.
14. The process of Claim 3, further comprising the steps of enzymatically oxidizing
the KDG by at least one oxidative enzyme to an oxidation product; and enzymatically
reducing said oxidation product by at least one reducing enzyme to 2-KLG.
10
15. A process for the non-fermentative production of 2-KLG from a carbon source,
comprising the following steps in any order, enzymatically oxidizing the carbon
source by at least one oxidative enzymatic activity to an oxidation product; and
enzymatically reducing said oxidation product by at least one reducing enzymatic
activity to 2-KLG.
15
16. The process of Claim 15 wherein said carbon source is KDG.
17. The process of Claim 15 wherein said oxidative enzymatic activity requires an
oxidized form of an enzymatic co-factor and said reducing enzymatic activity requires
a reduced form of said enzymatic co-factor and wherein said oxidized form of said
co-factor and said reduced form of said co-factor are recycled between at least one
oxidizing step and at least one reducing step.
20
- 25 18. The process of Claim 15 comprising the following steps in any order:
- a. enzymatically oxidizing the carbon source by a first oxidative enzymatic
activity to a first oxidation product;
- b. enzymatically oxidizing the first oxidation product by a second oxidative
enzymatic activity to a second oxidation product;
- c. enzymatically oxidizing the second oxidation product by a third oxidative
enzymatic activity to a third oxidation product; and
30

- d. enzymatically reducing the third oxidation product by a reducing enzymatic activity to 2-KLG.
- 5 19. The process of Claim 18 wherein at least one of said first, second and third oxidative enzymatic activities requires an oxidized form of an enzymatic co-factor and said reducing enzymatic activity requires a reduced form of said enzymatic co-factor and wherein said oxidized form of said co-factor and said reduced form of said co-factor are recycled between at least one oxidizing step and the reducing step.
- 10 20. The process of Claim 19 wherein said first oxidative enzymatic activity requires an oxidized form of said enzymatic co-factor.
- 15 21. The process of Claim 18 wherein said carbon source is glucose and said first enzymatic activity is a glucose dehydrogenase activity.
- 20 22. The process of Claim 21 wherein said glucose dehydrogenase activity is obtainable from a bacterial, yeast or fungal source.
- 25 23. The process of Claim 22 wherein said glucose dehydrogenase activity is obtainable from a source including *T. acidophilum*, *Cryptococcus uniguttalatus* and *Bacillus* species.
- 26 24. The process of Claim 19 wherein each of said first, said second enzyme and said third enzyme is a dehydrogenase activity.
- 30 25. The process of Claim 19 wherein at least one of said first, said second, said third and said fourth enzymatic activities are immobilized.
- 26 26. The process of Claim 19 wherein at least one of said first, said second, said third and said fourth enzymatic activities are in solution.
27. The process of Claim 25 wherein said second enzyme is a GADH activity.

28. The process of Claim 25 wherein said third enzyme is KDGDH activity.
29. The process of Claim 25 wherein said fourth enzyme is a reductase activity.
- 5 30. The process of Claim 29 wherein said reductase activity is obtainable from a bacterial, yeast or fungal source.
31. The reductase activity of Claim 29 wherein said source includes Corynebacterium and Erwinia.
- 10 32. The process of Claim 31 wherein said reductase activity is 2,5 DKG reductase.
33. The process of Claim 18 wherein said first oxidation product is gluconate, said second oxidation product is 2-KDG, and said third oxidation product is 2,5-DKG.
- 15 34. The process of Claim 18 that proceeds in an environment comprising recombinant host cells.
35. The process of Claim 34 wherein said host cell is viable.
- 20 36. The process of Claim 34 wherein said host cell is non-viable
37. The process of Claim 34 wherein said recombinant host cells comprise members of Enterobacteriaceae.
- 25 38. The process of Claim 34 that proceeds in an environment comprising recombinant host cell membranes and wherein at least one of said first, said second and said third enzymes are bound to said host cell membranes.
- 30 39. The process of Claim 37 wherein said recombinant host cell is a Pantoea species.

40. The process of Claim 39 wherein said recombinant host cell is *Pantoea citrea*.
41. The process of Claim 40 wherein said recombinant host cell has a mutation of at least one naturally occurring dehydrogenase activity.

5

42. The process of Claim 41 wherein said mutation is in a membrane bound GDH activity.

- 10 43. The process of Claim 41 wherein said host cell further comprises nucleic acid encoding a heterologous GDH activity.

44. The process of Claim 43 wherein said heterologous GDH activity is obtainable from *T. acidophilum*, *Cryptococcus uniguttalatus*, or a *Bacillus* species.

- 15 45. The process of Claim 18 wherein said oxidized form of said enzymatic cofactor is NADP+ and said reduced form of said enzymatic cofactor is NADPH.

46. The process of Claim 18 wherein said oxidized form of said enzymatic cofactor is NAD and said reduced form is NADH.

20

47. The process of Claim 1, Claim 15 or Claim 18 that is continuous.

48. The process of Claim 1, Claim 15 or Claim 18 that is batch.

- 25 49. The process of Claim 1, Claim 15 or Claim 18 that proceeds in an environment comprising organic solvents.

50. The process of Claim 1, Claim 15 or Claim 18 that proceeds in an environment comprising long polymers.

30

51. The process of Claim 14, Claim 15 or Claim 18 further comprising the step of obtaining ASA from said 2-KLG.

52. A host cell comprising nucleic acid having a mutation in the gene encoding GHD activity.
53. A host cell comprising nucleic acid having a mutation in the gene encoding KDGDH activity.
54. The host cell of Claims 52 or 53 that is a *Pantoea* species.
55. The host cell of Claim 52 further comprising nucleic acid encoding a heterologous GDH activity.
56. The host cell of Claim 55 further comprising nucleic acid encoding a heterologous reductase activity.
- 15 57. The process of Claim 1 optionally comprising the step of recovering said KDG or DKG.
58. The process of Claim 14, Claim 15 or Claim 18 wherein said 2-KLG is further purified via electrodialysis.
- 20 59. The process of Claim 45 or Claim 46 wherein said co-factor is purified via nanofiltration.
60. The process of Claim 18 that proceeds in an environment comprising salt.
- 25 61. The process of Claim 60 wherein the salt includes ammonium sulfate, sodium acetate, ammonium acetate, ammonium chloride, sodium sulfate, potassium phosphate, sodium phosphate, sodium chloride, , KCl, NH₄Cl, K₂SO₄ and NaI.
- 30 62. The process of Claim 60 that comprises a salt concentration between 0 mM and 500mM.

ABSTRACT

The present invention relates to non-fermentative methods for the production of ASA intermediates, KDG, DKG and KLG and methods for the regeneration of co-factor. The invention provides genetically engineered host cells comprising
5 heterologous nucleic acid encoding enzymes useful in the process.

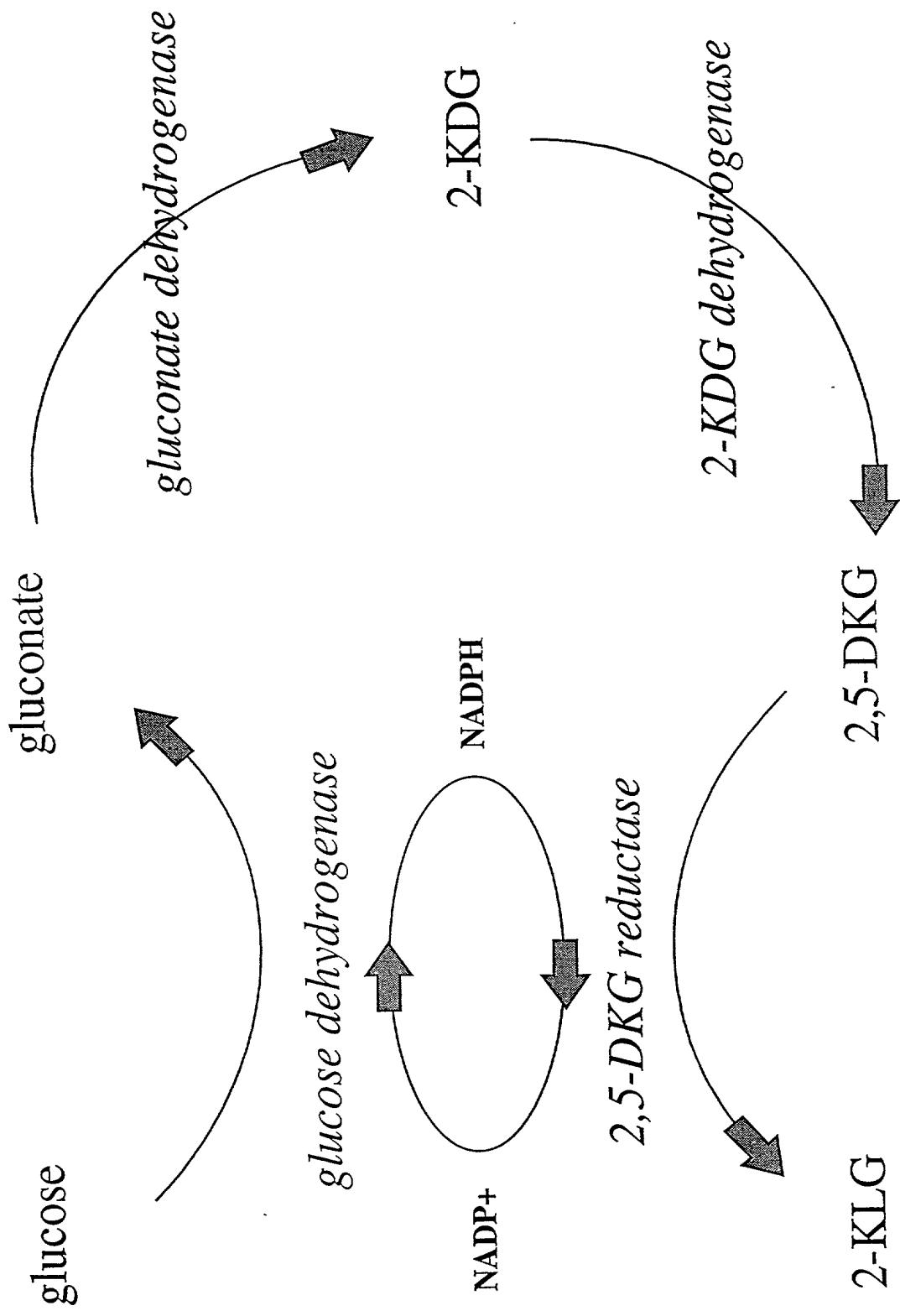


FIGURE 1

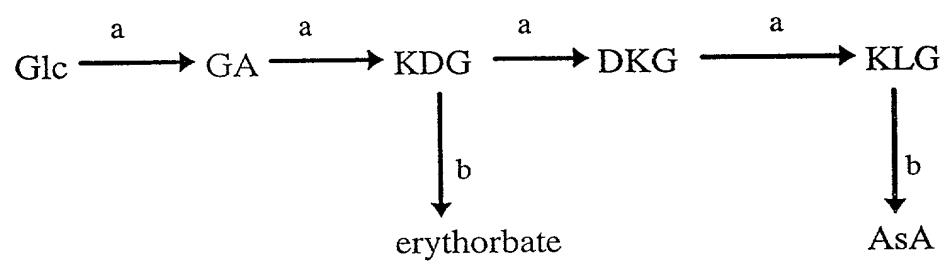


FIGURE 2

Reductase Activity in MeOH

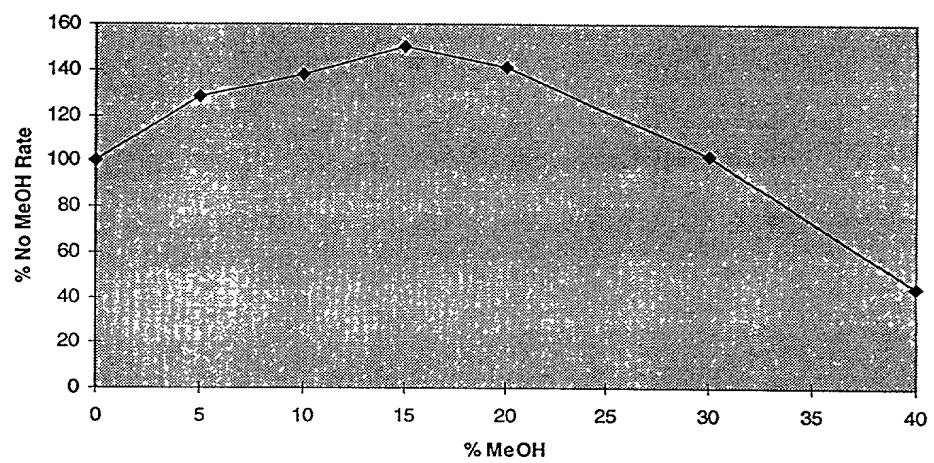


FIGURE 3

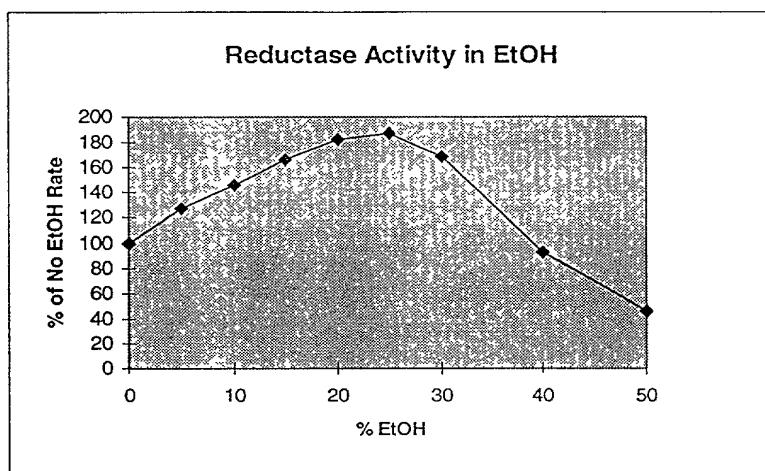


FIGURE 4

Reductase Activity in the Presence of Salt

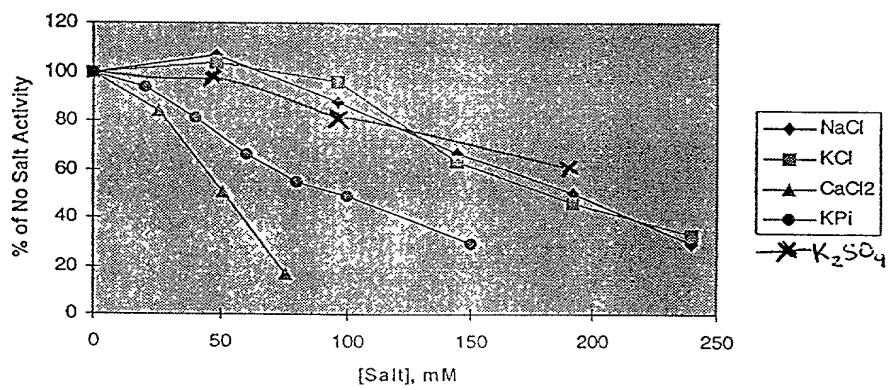


FIGURE 5

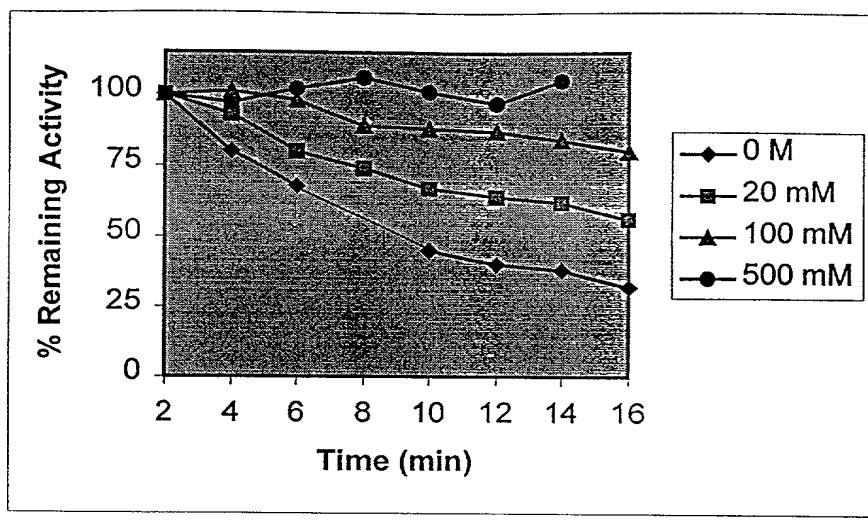


FIGURE 6

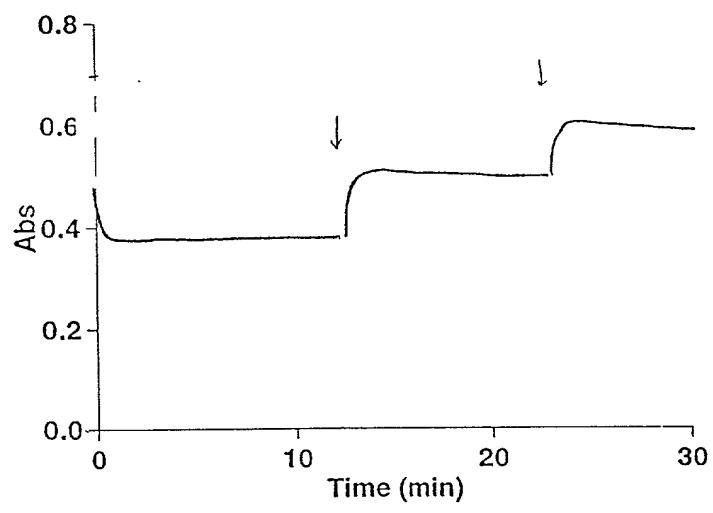


FIGURE 7

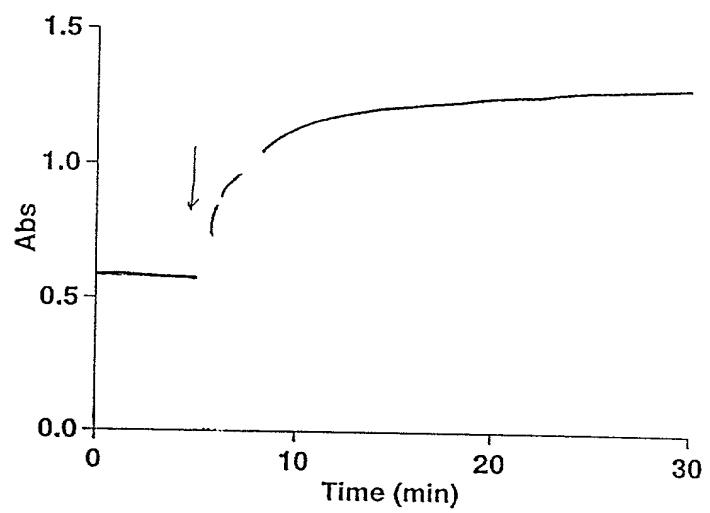


FIGURE 8

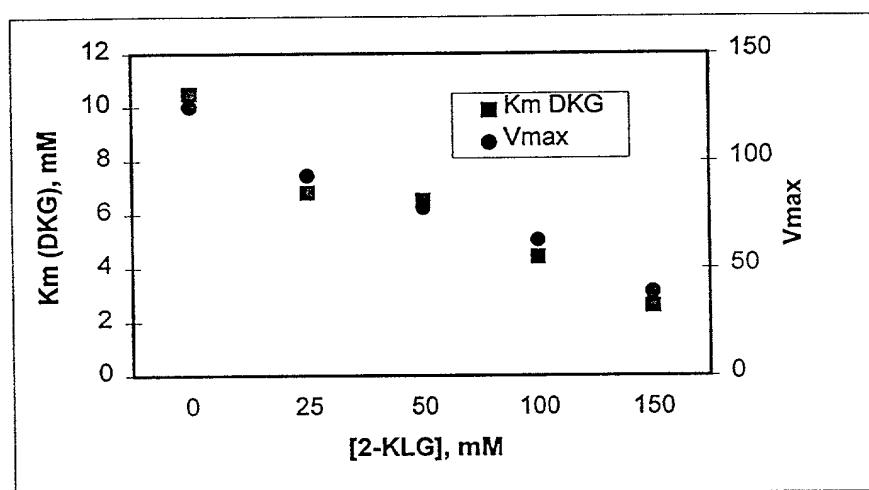


FIGURE 9

DECLARATION
AND POWER OF ATTORNEY

ORIGINAL APPLICATION

ATTORNEY DOCKET NO. GC538-2

AS A BELOW NAMED INVENTOR, I HEREBY DECLARE THAT:

MY RESIDENCE, POST OFFICE ADDRESS AND CITIZENSHIP ARE AS STATED BELOW NEXT TO MY NAME. I BELIEVE I AM A JOINT INVENTOR OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED **METHOD FOR PRODUCING ASCORBIC ACID INTERMEDIATES**, THE SPECIFICATION OF WHICH

CHECK ONE:

IS ATTACHED HERETO

WAS FILED ON _____ AS APPLICATION SERIAL NO. _____

I HEREBY STATE THAT I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE. I ACKNOWLEDGE THE DUTY TO DISCLOSE INFORMATION WHICH IS MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS §1.56.

I HEREBY CLAIM FOREIGN PRIORITY BENEFITS UNDER TITLE 35, UNITED STATES CODE §119, OF ANY FOREIGN APPLICATION(S) FOR PATENT OR INVENTOR'S CERTIFICATE LISTED BELOW AND HAVE ALSO IDENTIFIED BELOW ANY FOREIGN APPLICATION FOR PATENT OR INVENTOR'S CERTIFICATE HAVING A FILING DATE BEFORE THAT OF THE APPLICATION ON WHICH PRIORITY IS CLAIMED.

APPLICATION NUMBER	COUNTRY	DATE OF FILING	PRIORITY CLAIMED YES NO

I HEREBY CLAIM THE BENEFIT UNDER TITLE 35, UNITED STATES CODE §120, OF ANY UNITED STATES APPLICATION(S) LISTED BELOW AND, INSOFAR AS THE SUBJECT MATTER OF EACH OF THE CLAIMS OF THIS APPLICATION IS NOT DISCLOSED IN THE PRIOR UNITED STATES APPLICATION IN THE MANNER PROVIDED BY THE FIRST PARAGRAPH OF TITLE 35, UNITED STATES CODE §112, I ACKNOWLEDGE THE DUTY TO DISCLOSE MATERIAL INFORMATION AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS §1.56(A) WHICH OCCURRED BETWEEN THE FILING DATE OF THE PRIOR APPLICATION AND THE NATIONAL OR PCT INTERNATIONAL FILING DATE OF THIS APPLICATION.

APPLICATION NUMBER	DATE OF FILING	STATUS - PATENTED, PENDING OR ABANDONED
09/218,700	DECEMBER 22, 1998	PENDING

POWER OF ATTORNEY: AS A NAMED INVENTOR I HEREBY APPOINT AS MY ATTORNEY(S) WITH FULL POWER OF SUBSTITUTION AND REVOCATION, TO PROSECUTE THIS APPLICATION AND TRANSACT ALL BUSINESS IN THE PATENT AND TRADEMARK OFFICE CONNECTED THEREWITH:

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CHRISTOPHER L. STONE, REG. NO. 35,696
DEBRA J. GLAISTER, REG. NO. 33,888
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JEFFERY D. FRAZIER, REG. NO. 34,601

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201

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202

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			ZIP CODE 94131

I FURTHER DECLARE THAT ALL STATEMENTS MADE HEREIN OF MY OWN KNOWLEDGE ARE TRUE AND THAT ALL STATEMENTS MADE ON INFORMATION AND BELIEF ARE BELIEVED TO BE TRUE; AND FURTHER THAT THESE STATEMENTS WERE MADE WITH THE KNOWLEDGE THAT WILLFUL FALSE STATEMENTS AND THE LIKE SO MADE ARE PUNISHABLE BY FINE OR IMPRISONMENT, OR BOTH, UNDER SECTION 1001 OF TITLE 18 OF THE UNITED STATES CODE , AND THAT SUCH WILLFUL FALSE STATEMENTS MAY JEOPARDIZE THE VALIDITY OF THE APPLICATION OR ANY PATENT ISSUING THEREON.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202
DATE	DATE